

Review

Tumour heterogeneity and clonality — an old theme revisited

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A clone is defined as a population of cells derived through mitotic division of a single somatic cell of origin. Accordingly, a tumour represents the clonal progeny of a transformed somatic cell that has undergone somatic gene mutations [1–3]. Several methods are available nowadays to study tumour clonality, including immunogenotyping in lymphoid tumours, assessment of clonal loss of heterozygosity in many different neoplasms or clonal X-inactivation analysis in cellular proliferations of female patients (reviewed in [3]). At a superficial glance one might assume that a clonal tumour would necessarily exhibit a homogeneous genetic composition with regard to such genetic alterations. However, tumours are typically very heterogeneous in different respects. Look at a stained histological section of a tumour, and you will notice that its morphological appearance changes between areas as does the expression of immunological markers. Look at a leukaemic karyotype, and you will notice that in addition to typical marker chromosomes present in most if not all mitotic figures, additional non-random chromosomal abnormalities might be present in smaller numbers of cells, indicating the presence of distinct subpopulations. Cells belonging to a tumour may vary greatly in their capacity to metastasize, and a parent tumour, albeit clonal, is heterogeneous, containing various subclones with different metastatic potentials [4–7]. How, one wonders, can the concept of tumour clonality be reconciled with the notion of tumour heterogeneity?

It is important to return to the definition of a clone, which clearly does not imply that a tumour must be genetically or phenotypically homogeneous. A tumour is thought to arise from a single somatic cell present in normal tissue which, through the acquisition of gene mutations, has been turned into an early founder cell of the tumour clone (Figure 1). Examples of such early events are the BCR-ABL translocation in chronic myelogenous leukaemia (CML) which, if introduced into normal murine haematopoietic stem cells, is sufficient to launch a myeloproliferative disorder in mice [8, 9]. In colo-rectal cancers mutations in the familial

adenomatous polyposis (FAP) gene may turn up in dysplastic aberrant crypt foci and in very early colo-rectal adenomas assuming a gate-keeper role in tumorigenesis [10]. Within such 'founder' clones daughter cells will subsequently acquire additional mutations and will thus be equipped with additional chances of selection and proliferative advantages [1, 11, 12]. They would thus establish their progeny as subclones derived from a proximate parent clone and may in turn become parent clones themselves [13, 14]. Colo-rectal cancers have become a citation classic in this respect [10, 15]. The pathway from normal intestinal mucosa via adenoma to infiltrative and finally metastatic carcinoma is plastered with step-wise accumulation of somatic mutations which may hit many different genes (including the p53 tumour suppressor gene, a gene called *Deleted in Colo-rectal Carcinoma* [DCC], and many others). Before discussing the clonal composition of a tumour it would therefore be essential to define the mother cell that forms its starting point, which is recognized by a particular clonal marker. Within a model of multi-step accumulation of gene mutations in tumour development, a clone is allowed to begin wherever one wishes it to do so with the proviso that any such wishes would have to be clearly stated [3, 13, 14]. To be more specific: we may choose as the founder cell of a clone a member of another clone with an earlier starting point, in which case the clone of interest would be a subclone of the earlier one. This concept is important in carcinogenesis and provides the clue for reconciling tumour clonality and heterogeneity (Figure 1). An analysis of the clonal composition of tumours must therefore take into account the following aspects:

- a) an indication of the founder cell detected by a particular marker used for assessing tumour clonality;
- b) the spatial distribution of subclones within a tumour, particularly in solid tumours;
- c) clonality assessment may only provide information about the situation at the time of the analysis.

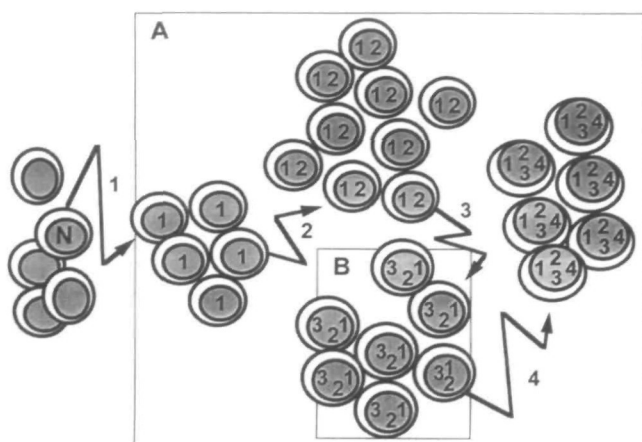


Figure 1. A schematic representation: The clonal evolution of a human tumour governed by multistep accumulation of somatic mutations. A normal somatic cell N (for example, a mucosal cell in the gut) may be hit by a somatic mutation (arrow 1) and starts to expand clonally. A cell present in this initial clone 1 may acquire an additional mutation (arrow 2), and subclone 2 which is related to, but distinct from, the initial clone 1 may emerge. Subsequent mutations (marked 3 and 4) may contribute to yet further heterogeneity within this tumour where the initial mutation 1 marking the founder clone is nevertheless retained. Molecular assessment of the clonal composition of this tumour may yield different results, depending on the time of analysis, the sample selected and the marker battery used. If samples A comprising all subclones and samples B (a tumour 'excerpt') are studied with marker 1, both tumour samples will appear monoclonal and homogeneous. If sample B is analysed with marker 4, the result will be negative, since this subclone does not contain mutation 4. If the detection of mutation 4 provides important prognostic information, sample B may not be representative of the tumour and the prediction of the patient's outcome.

The clonal composition of a tumour may change in the course of its history.

The simple corollaries of these principles are often ignored in the interpretation of molecular assessment of tumour clonality. It seems useful to examine a few selected examples in order to recognize the impact of molecular tumour heterogeneity on conclusions to be drawn from DNA analyses using molecular tumour markers.

Multiple tumour lesions in an organ: Derived from one or several different founder cells?

A variety of tumours typically present as multifocal lesions rather than as a contiguously spreading tumour mass. Examples are multifocal breast carcinoma, cancers in the urinary bladder, ovarian cancer involving the peritoneal cavity or hepatocellular carcinoma. It is often impossible morphologically to distinguish between simultaneously occurring tumours of independent origin, and tumour lesions with a common clonal 'mother' focus whose progeny subsequently spread in a multifocal fashion. Genetic markers assessing clonality of such lesions in a comparative way have thrown some interesting light on this problem.

In patients with urinary bladder cancer multiple tu-

mours scattered over the bladder epithelium are often identified at cystoscopy. Such observations have given rise to the concept of 'field defects' or 'field cancerisation' whereby carcinogenic agents would affect many different susceptible cells, resulting in their more or less simultaneous transformation. This in turn would lead to the development of multiple clonally distinct tumours. Conversely, multifocal tumours might have arisen via spread of tumour cells derived from a common progenitor lesion. There is no way to differentiate between these two options by morphological criteria. Multifocal tumour lesions in the bladder of a given patient often contain identical patterns of clonal X-inactivation, and show loss of the same allele on chromosome 9q where an as yet unidentified tumour suppressor gene operative in early bladder cancer development is thought to reside [16, 17]. These findings suggest a common clonal origin of multifocal bladder tumour lesions rather than an independent, truly multicentric origin. In the same tumours a search for loss of heterozygosity on chromosomes 17p and 18q, however, may reveal patterns that in a given patient differ between geographically separate lesions, indicating disparate clonal evolution in such daughter tumours.

Ovarian cancer has been studied in a similar fashion. Using p53 gene mutations as a marker, evidence for a common clonal origin of ovarian cancer presenting with multifocal involvement of the peritoneum has been obtained [18, 19]. A different genetic marker system, for example DNA fingerprinting, may demonstrate that within an individual patient the primary ovarian tumour and peritoneal metastases share a basic clonal DNA fingerprint pattern with additional variations between different sites, indicating different tumour subpopulations derived from a common stem line [18–21]. In contrast, papillary serous carcinoma of the peritoneum which is morphologically indistinguishable from papillary serous carcinoma of the ovary may represent a truly multifocal tumour, at least in some cases [22].

Hepatocellular carcinoma sometimes presents with multiple tumour nodules in the liver, posing the differential diagnosis between cancers of truly multicentric and those of metastatic origin within the liver. Depending on the molecular marker used, either possibility seems to occur. Clonal p53 gene mutations [23] or DNA fingerprint alterations [24] may either be shared by all lesions, indicating a common clonal origin with subsequent spread, or may show heterogeneous patterns.

An interesting problem is the clonal analysis of multicentric/multifocal breast cancer or assessment of clonality in ductal carcinoma *in situ* (DCIS) and invasive ductal cancer present in the same breast. Allelic loss identified in DCIS lesions usually persists in the synchronous invasive ductal breast cancer foci and regional lymph node metastases [25], indicating that in all likelihood the invasive component is derived from the DCIS. LOH at other loci, for example at 11p, may,

however, be restricted to invasive cancer and may thus represent a later event on the pathway to invasion. Several reports on multifocal/multicentric invasive breast cancer have either confirmed [26] or refuted [27] the notion that such lesions represent truly independent, separate clonal tumours.

Differences in the choice of DNA markers must be appreciated when interpreting such analyses. The detection of a clonal DNA marker common to all areas of a tumour or a molecular tag consistently present in several anatomically separate tumour lesions in an organ may duly be interpreted as evidence of their common clonal origin. For example, assessment of clonality by X-inactivation analysis is based on inactivation of either the paternal or the maternal X-chromosome copy in a given cell and thus represents a marker which preexists in early transformed founder cells of malignant tumours, since X-inactivation patterns in particular cells or tissues are established in early embryogenesis. Therefore, clonal X-inactivation analysis provides insight into an early phase of carcinogenesis and represents an 'early marker' of the clonal relation of different tumour lesions [28]. If a marker captures a 'late' genetic event, it is conceivable that separate tumour lesions in an organ show disparate patterns whilst still being clonally related by virtue of sharing identical X-inactivation patterns. For example, clonal loss of heterozygosity traced by polymorphic DNA markers (variable-number-of-tandem-repeat markers or microsatellites) may occur at any stage of a multi-step accumulation of genetic mutations in an evolving cancer [10, 15, 23, 29]. Heterogeneity with respect to such a DNA marker between various synchronous but geographically separate tumour lesions is no definite proof of their truly multifocal and independent clonal origin, since such tumours, although genetically heterogeneous, might still be clonally related as subclones of a common lesion of origin [16]. In summary, multifocal/multicentric tumour lesions in an organ are often clonally related, and molecular heterogeneity between various such foci is no definite proof of their clonally independent development.

Post-transplantation lymphoproliferative disorders (PT-LPD) may be an interesting exception to this rule of thumb [30–35]. These tumours often present as synchronous multiple lymphomas, mostly of B-cell phenotype. If separate PT-LPD lesions of an immunosuppressed transplant patient are examined for their clonal composition with appropriate markers such as Ig gene rearrangements or patterns of Epstein-Barr virus terminal-repeats [36], distinct patterns will be noted with respect to both markers. Separate PT-LPD lesions occurring synchronously in a single organ or patient may often represent multiple independent primary lymphoid proliferations rather than haematogeneous spread of a single clone disorder as in 'conventional' malignant lymphoma.

Hodgkin's disease (HD) has always presented a particularly intriguing problem with respect to clonality. A

recent report focussed on the detailed analysis of rearranged immunoglobulin variable-region heavy-chain (VH) genes in single Reed-Sternberg cells picked under microscopic control from lymph nodes involved with Hodgkin's lymphoma [37]. Assessment of tumour heterogeneity was thus brought down to the single cell level. Three patterns were observed: identical VH rearrangements in all cells from a HD lymphoma, indicating a common monoclonal origin of these Reed-Sternberg cells, possibly from a naive or memory B-cell; unrelated and unique VH rearrangements, indicating a polyclonal origin of these cells; and a combination of the two. These findings point to a possibly distinct pathogenesis of either polyclonal or monoclonal HD.

The spatial distribution of subclones within a tumour, molecular tumour diagnosis and the pitfall of sampling

Molecular genetic abnormalities in tumours are of immense interest in basic science, but molecular markers are also being increasingly used in translational research and in the clinic. In many common cancers we are still looking for improvement in assessing the prognosis of individual cases in the hope of being able to tailor management, particularly treatment. Particular gene mutations are not simply surrogate markers for biological behaviour but often represent the actual driving forces underlying neoplastic growth. One thus hopes that their detection would refine and sharpen our diagnostic armamentarium, because molecular tumour typing would permit the sorting out of defined tumour entities within morphologically defined groups of tumours of heterogeneous molecular composition. Let us pick a frequent and well studied example: colorectal adenocarcinomas presenting in Dukes stage B without discernible involvement of regional lymph nodes comprise a mixed bag of cases with rather variable clinical courses. The study of these cancers for loss of heterozygosity (LOH) with polymorphic microsatellite markers from the long arm of chromosome 18 (18q) shows that slightly less than half of them show LOH (18q-) and the remainder retain a normal chromosome 18q status. The presence or absence of this marker appears to be highly correlated with outcome, since the survival rate of patients with Dukes B / 18q- tumours is similar to that of patients with Dukes C node-positive disease [38]. Patients with Dukes B tumours which retain a normal 18q configuration enjoy a significantly better outcome. In practice such molecular analyses are often performed on single small biopsy specimens taken from large lesions. Interpretation of the results must take into account the question of whether a tumour specimen is truly representative of the entire tumour. This is the point where molecular tumour heterogeneity due to clonal evolution comes in. 'Early' mutations, for example those with a gate-keeper function in tumour development, may be expected to

be present throughout the tumour because they must have arisen in an early founder cell. Mutations restricted to particular tumour subclones, however, are not necessarily present throughout the cancer but possibly restricted to particular geographical tumour areas. We looked at this problem in a series of gastro-intestinal cancers taken from female patients whereby several geographically distinct tumour areas were examined with a battery of clinically relevant molecular markers [39]. The question of whether these tumours had arisen from one or perhaps a few single mucosal founder cells was tackled by clonal X-inactivation analysis [28]. Each tumour (with one exception) showed a uniform clonal X-inactivation pattern throughout all of the samples, suggesting the cancer's origin in a single early mucosal founder cell. However, LOH, for example at 18q and clonal microsatellite mutations due to replication errors in RER⁺ tumours [40–44] often differed considerably between various morphologically defined areas from a given tumour, indicating considerable clonal heterogeneity with respect to these markers. These mutations most probably arose from an ancestral cell that was already present in the tumour and in turn became the founder of a particular subclone identified by these molecular tags. Molecular analyses based on such markers should be interpreted with caution because sampling errors in cancers with clonal intratumour heterogeneity will affect the results. Tumour subclones bearing a particular marker may be missed in the specimen selected for analysis and yet they may behave differently from tumour tissue represented in the biopsy analysed [39, 45]. Accordingly, metastatic foci of such cancers, for example in the liver, may harbour molecular abnormalities that are not readily detectable throughout the corresponding primary tumours [46].

In prostate cancer radical prostatectomy specimens provide an opportunity to look at the intratumour clonal composition of this common type of tumour. In addition to dominant cancer nodules, separate smaller tumour foci are often observed to exhibit different histological grades. Within a prostate cancer there may be tumour subpopulations which manage to implant their progeny into regional lymph nodes whilst other subclones such as foci of well differentiated carcinoma may be indolent. In addition, lesions representing prostatic intraepithelial neoplasia (PIN) which correspond to non-invasive *in situ* tumour lesions may be present. Microdissection of prostate cancer specimens permits separate molecular mapping of such diverse lesions and their genetic markers [47, 48]. Candidate genetic markers include deleted gene sequences or LOH on chromosome 8p, 10q and 16q. A detailed molecular analysis will reveal that the morphological heterogeneity in prostate neoplasias and premalignant lesions is reflected at the molecular level. Separate PIN lesions may show different clonal patterns of allelic loss at 8p12–21, suggesting that they may arise independently of one another [48, 49]. When comparing metastatic cancer foci in regional lymph nodes with various indi-

vidually microdissected lesions in a prostate, the molecular genetic relationship of metastatic tumour to one or the other of the primary tumour foci may be traced. It is of interest to note that allelic loss patterns may not always identify the morphologically-dominant primary prostate tumour as the source of metastases; on occasion such genetic links rather point to additional tumour foci as the origin of lymph node metastases.

Looking at a snapshot when really you should watch a film

The clonal composition of a neoplasm must be viewed as a dynamic process which may undergo considerable alteration over time. The analysis of a solid tumour or a haematological neoplasm with an appropriate battery of molecular markers can only be interpreted correctly when the effects of clonal evolution leading to molecular heterogeneity of the tumour are duly appreciated. Leukaemias harbour specific (even individual-specific) clonal markers which are amenable to amplification with the rapid and sensitive polymerase-chain reaction (PCR). If the 'traditional', relatively insensitive means of following patients treated for leukaemia are used to define remission, a considerable undetected leukaemic burden usually remains in the patient's organism which will ultimately result in clinical relapse. The PCR now offers a much more sensitive follow-up by amplifying leukaemia-specific markers such as the BCR-ABL transcript in CML [50, 51], or the fusion messenger RNA derived from the DNA stretch bridging chromosome 15 (bearing the so-called *myl*/PML gene) and chromosome 17 (harbouring the retinoic acid receptor α gene) in acute promyelocytic leukaemia [52–55]. In acute lymphoblastic leukaemia (ALL) individual-specific clonal immunoglobulin (Ig) or T-cell receptor gene rearrangements may provide templates for preparing patient-tailored molecular probes which can pick up minimal numbers of residual leukaemic cells in remission marrow [56–67]. Successful follow-up using molecular markers depends on the question of whether leukaemic cells would retain a stable composition of diagnostic molecular markers or whether clonal evolution within the original leukaemic clone would eventually lead to molecular heterogeneity and diagnostic difficulties, particularly false-negative results [58, 67–69]. In CML the BCR-ABL fusion gene provides a 'fil rouge' which can be traced through the course of disease. An interesting case of CML in a patient who developed myeloid blast crisis after six years of chronic phase was recently reported [70]. Whilst the BCR-ABL marker which had been present throughout the chronic phase still persisted, a considerable population of malignant cells in blast crisis had acquired a new clonal marker, a point mutation in the p53 tumour suppressor gene. The patient responded to chemotherapy and a second chronic phase was induced in which the p53 mutation was no longer detectable in

the malignant blasts. A few months later, there was a second blast crisis, apparently involving a different CML subclone, since the p53 mutation that had marked the first blast crisis remained undetectable. The original clonal BCR-ABL marker still remained. This case is a good example of the fact that the clonal composition of a neoplasm may change over time, with new clonal markers coming up at defined stages which may either persist or vanish again. The molecular marker pattern of a neoplasm can be flexible and heterogeneous in a time-dependent manner. Assessment of clonality with a particular marker will yield different results, depending on when the analysis is made.

Molecular follow-up of patients with acute lymphoblastic leukaemia provides another example of how the dynamics affecting gene mutations in cancer over time may affect molecular diagnostics. In each patient Ig gene rearrangements in the malignant lymphoid cells are clone-specific and contain unique base sequences. It has been suggested that these can be cloned and then used as custom-tailored probes for the sensitive detection of minimal residual leukaemia in remission marrow [61–68]. However, Ig gene rearrangements are not necessarily stable in the leukaemic cells and gene sequences may change because of ongoing somatic mutations. For example, accumulation of point mutations and insertion of extra nucleotides in Ig gene variable regions may be extensive [71–74]. As a consequence, leukaemic subclones emerging at relapse will not entirely reflect the clonal composition of malignant cells present at diagnosis. In practice, such subclones will not be detected with clone-specific probes prepared from diagnostic specimens obtained at presentation.

Lymphomas of low grade malignancy often convert into lymphomas of more aggressive clinical behaviour which on morphology may look quite distinct from samples obtained at initial diagnosis. For example, follicular lymphoma may evolve into diffuse large-cell lymphoma. Assessment of the clonal relationship between such morphologically distinct lesions seen in the same patient nicely illustrates that the study of their molecular composition must reckon with molecular heterogeneity. Results will therefore depend on which DNA markers are used at a given time. Follicular lymphoma is usually characterized by the translocation t(14;18) which fuses parts of the Ig heavy-chain gene on chromosome 14 with the BCL-2 gene on chromosome 18. Analysis of the bridging DNA sequences at the t(14;18) junction reveals that this marker usually persists throughout the course of the disease, including transformation into a highly malignant lymphoma [73–77]. By simply looking at this marker, one might erroneously conclude that the molecular composition of the original clone remains unchanged. Analysis of a second clonal marker, i.e., rearranged Ig heavy-chain genes, reveals a molecular model to mirror transformation identified by morphology. The initial clone (a mother clone, as it were) remains stably marked by the

t(14;18) translocation present in its founder cell. As a result of ongoing somatic hypermutation in rearranged Ig genes, the B-cell tumour population nevertheless becomes heterogeneous with individual clonally related subclones tagged by specific rearranged Ig variable regions [73–80]. Serial analysis of such sequences permits the construction of a genealogical tree to portray the clonal relationship present in a lymphoid neoplasm tracing subclones which are evolutionally related, derived from a common progenitor, but which still differ because of different maturational histories. Some subclones can be selected through therapy, or can overrule others via a proliferative selection advantage [71, 74, 75]. In our example, the notion is that a transformed malignant subclone presenting as large-cell lymphoma, arises from the original follicular lymphoma clone rather than representing the product of an independent transforming event.

A recent detailed clonal analysis of Hodgkin's disease of the lymphocytic-predominance subtype by immunogenotype PCR revealed a clonal relationship between various morphologically-related lesions [81]. Thus, in a lymph node, progressively transformed germinal centres may represent precursor lesions of lymphocytic-predominance HD. They are mostly polyclonal and only a few of them become founder cells of oligoclonal lesions morphologically recognized as lymphocytic-predominance HD. This state may persist for years, but eventually additional genetic events may act on one or the other of the B-cell subclones and cause its preferential expansion and a monoclonal lymphoid proliferation that appears on morphology as a monoclonal B-cell large-cell lymphoma.

These principles are not restricted to haematological neoplasms, but apply also to the solid tumours. Gliomas may perhaps serve to illustrate this point. Astrocytomas (low-grade tumours) may evolve into faster growing, more aggressive and invasive tumours, glioblastomas. This tumour progression may be associated with the acquisition of clonal mutations within the p53 gene. If low-grade tumours are carefully analysed, subpopulations of cells may be found that already contain such p53 gene mutations which become predominant in glioblastoma [82, 83]. A rare cell in an 'early' lesion carrying a specific change may thus become the dominant cell type as the tumour progresses [84].

Conclusions

Molecular assessment of the clonal composition of human neoplasms represents much more than just a 'complicated way of proving the obvious fact that tumours are monoclonal'. A successful analysis must take into account the geographical selection of tissue samples, the choice of the DNA markers used to tackle the problem, and their hierarchical position within a chain of accumulated gene mutations and the time-point in carcinogenesis when a tumour sample is har-

vested. Clonal molecular tumour heterogeneity might lead to sampling errors and thus to erroneous interpretations of molecular analyses. Tumour subclones emerging at relapse may not entirely reflect the clonal composition of malignant cells present at diagnosis and will not necessarily be detected with tumour-specific markers suitable for pinpointing a tumour at the time of presentation [39, 85, 86]. Furthermore, clonal tumour heterogeneity must be considered when 'molecular' treatment strategies, such as knocking out the expression of key genes through antisense oligonucleotides, molecular mending, sewing and stitching of crucial gene mutations in cancer cells, and other concepts of gene therapy [87, 88] are designed. The recognition of molecular tumour heterogeneity, indicating the coexistence of clonally-related subclones within a tumour of common clonal origin is thus important both theoretically and practically.

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